The Role of Oxidative DNA Damage in Human Arsenic Carcinogenesis: Detection of 8-Hydroxy-2′-Deoxyguanosine in Arsenic-Related Bowen's Disease

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Arsenic is widely distributed in nature in the form of either metalloids or chemical compounds, which cause a variety of pathologic conditions including cutaneous and visceral malignancies. Recently, reactive oxygen species have been hypothesized to be one of the causes of arsenic-induced carcinogenesis. 8-Hydroxy-2′-deoxyguanosine is one of the major reactive oxygen species-induced DNA base-modified products that is widely accepted as a sensitive marker of oxidative DNA damage. We studied the presence of 8-hydroxy-2′-deoxyguanosine by immunohistochemistry using N45.1 monoclonal antibody in 28 cases of arsenic-related skin neoplasms and arsenic keratosis as well as in 11 cases of arsenic-unrelated Bowen's diseases. The frequency of 8-hydroxy-2′-deoxyguanosine positive cases was significantly higher in arsenic-related skin neoplasms (22 of 28; 78%) than in arsenic-unrelated Bowen's disease (one of 11; 9%) ($p < 0.001$ by χ^2 test). 8-Hydroxy-2′-deoxyguanosine was also detected in normal tissue adjacent to the arsenic-related Bowen's disease lesions. Furthermore, arsenic was detected by neutron activation analysis in the deparaffined skin tumor samples of arsenic-related disease (four of five; 80%), whereas arsenic was not detected in control samples. Our results strongly suggest the involvement of reactive oxygen species in arsenic-induced human skin cancer. ***Key word: neutron activation analysis. J Invest Dermatol 113:26–31, 1999***

Arsenic is widely found in nature and is distributed throughout the environment by water. It has been used in various ways: as pesticides, in silicon water technology such as computer microchips, chemical weapons, and traditional forms of medicine. Epidemiologic studies have revealed that inorganic arsenic is carcinogenic for human organs, including the lung, liver, skin, bladder, and kidney (Yeh, 1973; Blot and Fraumeni, 1975; IARC, 1980; Schwartz, 1997). Arsenic-induced skin cancers or skin diseases in patients from some endemic areas in Taiwan, Thailand, and Japan (Chen *et al*, 1992; Imamura and Odaka, 1962) are quite common. Its carcinogenic mechanism, however, so far remains unclear.

An *in vitro* study has shown that arsenic induces chromosome aberrations and DNA-protein cross-links (IARC, 1980). Reactive oxygen species (ROS) produced by dimethylarsenic acid, one of the major metabolites of inorganic arsenics, were suggested to be involved in the generation of DNA damage (Yamanaka *et al*, 1989). Recently, Wanibuchi *et al* (1997) found that dimethylarsenic acid has the potential to promote rat liver carcinogenesis, possibly via a mechanism involving stimulation of cell proliferation and DNA damage caused by ROS. This prompted us to investigate the involvement of ROS in arsenic-induced human skin cancers.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is generated by hydroxyl radical (Kasai and Nishimura, 1984), singlet oxygen (Floyd *et al*, 1989; Devasagayam *et al*, 1991), or direct electron transfer, which does not involve the participation of any ROS (Kasai *et al*, 1992). 8-OHdG is considered to be one of the main oxidative base damage to DNA, and may cause mutation (G:C to T:A) at DNA replication (Shibutani *et al*, 1991). Currently, 8-OHdG is widely accepted as a sensitive marker of oxidative DNA damage and oxidative stress. In the present study, we examined whether neoplastic and precancerous skin lesions of arsenic-related individuals are oxidatively stressed using 8-OHdG as a marker. As UVB, especially *in vivo* (Hattori *et al*, 1997) and mainly UVA and visible light in the presence of photosensitizer (Kvam and Tyrrell, 1997; Rosen *et al*, 1997) participate in ROS formation, all skin materials were obtained from non-sun-exposed or less sun-exposed areas in order to minimize the effect of sun exposure upon 8-OHdG generation.

8-OHdG measurement by high-performance liquid chromatography using an electrochemical detector has been a widely accepted method for such an investigation. A large amount of DNA, however, is required for high-performance liquid chromatography–electrochemical detection analysis. As we could obtain only a small amount of paraffin-embedded blocks, we chose to carry out semiquantitative immunohistochemical measurement of 8-OHdG by determining the signal intensity using monoclonal antibody against 8-OHdG (N45.1). This method has been shown to correlate well with 8-OHdG levels by high-performance liquid chromato-

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Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; INAA, instrumental neutron activation analysis; ROS, reactive oxygen species.

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Table I. 8-OHdG detection in arsenic-related skin tumors

Cases	Age	Sex	Diagnosis[a]	Site	Characteristics of histopathology			
					Mitosis	Individual keratinization	Clumping cell	8-OHdG[b] detection
Non-exposed area								
1	68	M[d]	BD	abdomen	+	–	–	++
2		F[c]	BD	thigh	+	–	–	++
3	60	F[e]	BD	back	+	–	–	–
4	26	M[e]	BD	penis	++	–	–	+
5	59	F[d]	BD	abdomen	++	–	+	++
6	67	M[d]	BD	abdomen	+	–	+	++
7	63	M[c]	BD	scrotum	+	+	–	–
8	53	F[e]	BD	thigh	+	+	–	++
9	74	M[d]	BD	back	++	+	+	++
10	70	F[d]	BD	back	++	++	+	++
11	83	F[c]	BD	thigh	++	++	++	++
12	58	M[d]	BC	abdomen	+	–	+	+
13	72	M[d]	BC	abdomen	+	+	–	+
14		M[c]	BC	scrotum	+	+	–	+
15	79	M[d]	BC	back	+	+	–	++
16	60	M[e]	BC	penis	++	++	–	–
17	76	M[e]	BC	back	++	+++	+	–
Less-exposed area								
18	63	M[c]	AK	palm	–	–	–	–
19		F[c]	AK	palm	–	–	–	++
20		M[c]	AK	foot	+	–	–	+
21	60	M[c]	AK	dorsal hand	+	–	–	++
22	60	M[c]	AK	knee	+	–	–	++
23	61	M[e]	BD	chest	+	–	–	+
24	53	M[d]	BD	chest	+	–	+	–
25	83	F[c]	BD	palm	+	+	+	++
26	87	M[e]	BD	chest	+	+	+	++
27	75	M[e]	BD	chest	+	+	+	++
28	72	M[d]	BD	chest	+	++	+	++

[a]BD, Bowen's disease; BC, Bowen's carcinoma; AK, arsenic keratosis.
[b]Intensities of 8-OHdG staining were grouped into three grades (–, +, ++) according to the 8-OHdG index determination. See *Materials and Methods* for detail.
[c]Samples from Japan.
[d]Samples from Taiwan.
[e]Samples from Thailand.

graphy over the same samples (Toyokuni *et al*, 1997). We found that the frequency of 8-OHdG positive tumors is significantly higher in arsenic-induced epidermal neoplasms than in the arsenic-unrelated epidermal neoplasms.

Furthermore, we detected arsenic in some of the available tumor sections using instrumental neutron activation analysis (INAA) in view of its high sensitivity for the detection of arsenic (Molokhia and Portnoy, 1969). Possible involvement of ROS in arsenic-induced carcinogenesis will be discussed.

MATERIALS AND METHODS

Bowen's disease and arsenic keratosis Samples of arsenic keratosis, arsenic-induced Bowen's disease and arsenic-induced Bowen's carcinoma arising in non-sun-exposed or less sun-exposed areas were obtained from 28 individuals (age 26–83) from either Taiwan, Thailand, or Japan living in areas where chronic arsenicism was endemic (**Table I**). These patients revealed clinical features characteristic of arsenicism such as cutaneous melanosis, keratosis of palms and soles (arsenic keratosis) and multiple Bowen's disease and internal malignancy. Clinical features of the Japanese patients were reported in detail previously (Imamura and Odaka, 1962; Miki *et al*, 1982). As control tumor samples, arsenic-unrelated Bowen's disease and Bowen's carcinoma of non sun-exposed areas were obtained from 11 Japanese individuals. Bowen's disease is defined as "a persistent, progressive, nonelevated, red scaly or crusted plaque which is due to an intraepidermal carcinoma and is potentially malignant" (Mackie, 1992), and we defined Bowen's carcinoma as invasive squamous carcinoma developing from a Bowen's disease.

Immunohistochemistry Skin specimens were fixed in 10% neutral formalin, then embedded in paraffin, sliced at 3.5 μm, mounted on glass slides coated with poly L-lysine, and subjected to either hematoxylin/eosin or immunohistochemical staining using ABC method (Hsu *et al*, 1981). Sections were treated with microwave in 10 mM citrate buffer pH 6.0 with 0.1% polyoxyethylene sorbitan monolaurate (Wako, Osaka, Japan) for 5 min three times for antigen retrieval. Specimens were then incubated with normal rabbit serum (Dako, Kyoto, Japan; diluted to 1:75) for 30 min, then incubated with N45.1 monoclonal antibody (2.5 μg per ml) specific for 8-OHdG (Toyokuni *et al*, 1997) overnight at 4°C followed by incubation with biotin-labeled rabbit anti-mouse IgG serum (Dako; Diluted 1:300) for 50 min and finally with avidin–biotin alkaline phosphatase complex (Vector Laboratories, Burlingame, CA; diluted 1:100) for 40 min. Substrate for alkaline phosphatase was obtained from Vector.

Adsorption test To confirm the specificity of immunostaining of ROS-specific products, adsorption tests were performed by adding free antigens, 200 and 500 μM 8-OHdG (final concentration; Wako) on specimens during the incubation period of primary antibody. Serial sections were stained with the same procedures with or without free antigens and signal intensities were compared with each other.

Quantitation of immunohistochemical data The quantitation was carried out as described previously (Toyokuni *et al*, 1997). The following equation was used for the quantitation of immunohistochemical data:

$$\text{8-OHdG index} = \Sigma[(A - \text{threshold}) \times \text{area (pixel)}]/\text{total cell number}, \quad A > \text{threshold}$$

where A is the staining density indicated by a number on the gray scale. Specimens stained with hematoxylin and eosin were used for total cell calculation. Three color images of 35 mm slides for each 8-OHdG immunohistochemistry specimen were obtained as PICT files with a slide scanner (Polascan 35: Polaroid, Tokyo, Japan) and the brightness and contrast of each image file were enhanced by Adobe Photoshop version 3.0 followed by an analysis using NIH image freeware (version 1.59). Average 8-OHdG index of three to five experiments for each specimen was obtained. Grade of 8-OHdG detection was expressed as (–, +, ++)

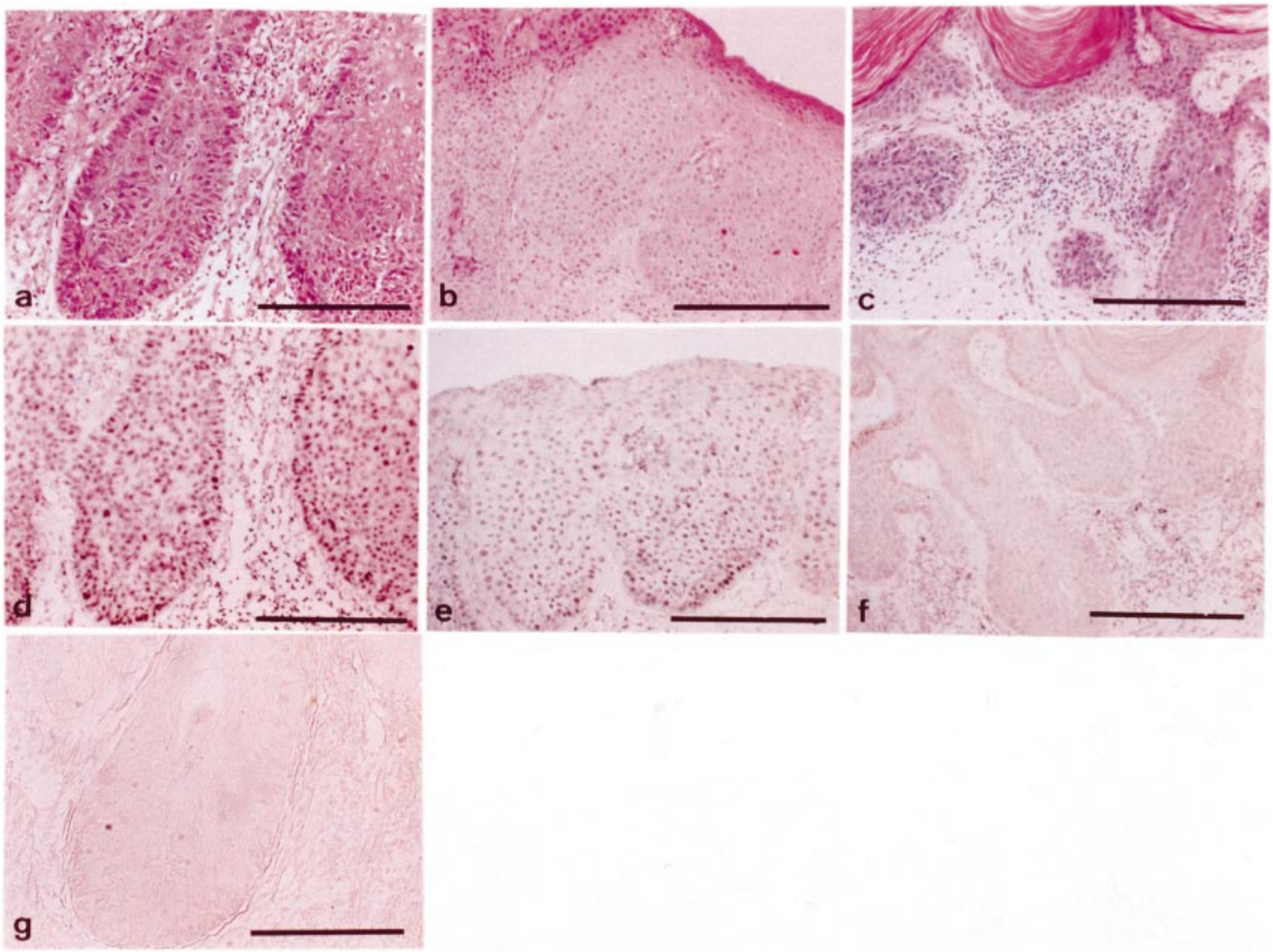

Figure 1. 8-OHdG immunostaining in arsenic related skin tumors. Representative 8-OHdG staining of ++, +, and – are shown in (*d*), (*e*), and (*f*), respectively. (*a*) Hematoxylin and eosin staining of arsenic-related Bowen's disease (case 9 in **Table I**). Acanthosis and atypical cells with hyperchromatic nuclei and mitotic figures are observed. (*b*) Hematoxylin and eosin staining of arsenic-related Bowen's disease (case 4 in **Table I**). Acanthosis, papillomatosis, parakeratotic layer are prominent. (*c*) Hematoxylin and eosin staining of arsenic-unrelated Bowen's disease (case 4 in **Table II**). Acanthosis, papillomatosis, thick hyperkeratotic and parakeratotic layer, and horn pearls are prominent. Mitotic cells are observed. (*d–f*) 8-OHdG immunostaining of using monoclonal antibody specific to 8-OHdG (N45.1) in the same samples as (*a–c*), respectively. 8-OHdG was detected in the nuclei of epidermal cells. Grade of 8-OHdG index was ++, +, –, respectively (**Table I**). *Scale bar*: 100 μm. (*g*) Adsorption test: Specimens of case 9 in **Table I** was stained exactly the same as (*d*) except that adding 200 μM 8-OHdG to specimens during the incubation period of primary antibody. N45.1 was neutralized by the excess free antigen and 8-OHdG in the specimen was not detected whereas 8-OHdG was detected in the absence of excess free antigen as shown in (*d*).

Table II. 8-OHdG detection in arsenic-unrelated skin tumors

					Characteristics of histopathology			
Cases	Age	Sex	Diagnosis[a]	Site	Mitosis	Individual keratinization	Clumping cell	8-OHdG[b] detection
1	71	M	BD	back	+	–	–	–
2	52	M	BD	plantar	+	–	–	–
3	82	M	BD	hip	+	+	–	–
4	74	F	BD	abdomen	+	+	+	–
5	61	F	BD	hip	+	+	+	–
6	82	F	BD	hip	+	+	+	–
7	68	M	BD	abdomen	+	+	+	–
8	59	F	BD	back	+	+	+	+
9	44	M	BD	back	+++	+	+	–
10	73	M	BD	hip	+++	++	+	–
11	88	M	BC	scrotum	+	+	–	–

[a]BD, Bowen's disease; BC, Bowen's carcinoma.
[b]Intensities of 8OHdG staining were grouped into three grades (–, +, ++) according to the 8-OHdG index determination. See *Materials and Methods* for detail.

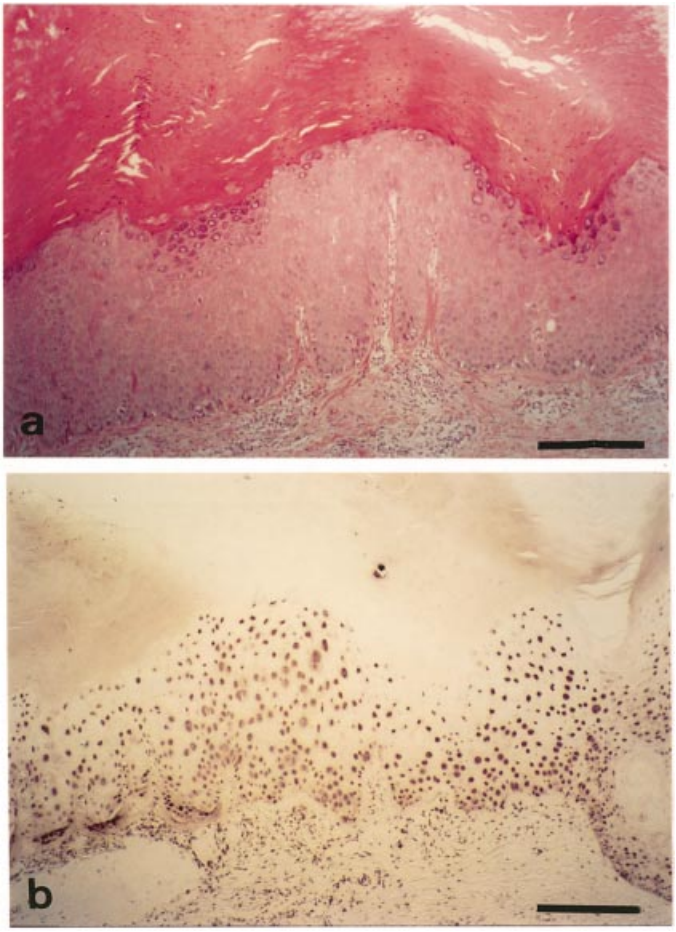

Figure 2. 8-OHdG staining of arsenic keratosis (case 22, hematoxylin–eosin staining). (*a*) Thick compact parakeratotic hyperkeratosis is overlying a mild keratotic dysplasia. Mitosis or atypical cells are not observed. (*b*) 8-OHdG staining of the same samples as (*a*). 8-OHdG is also detected in the nuclei of epidermal cells. *Scale bar*: 100 μm.

by the 8-OHdG index number (–, 0–10; +, 11–100; ++, >100), respectively.

Detection of arsenic by INAA In tumor samples which we could obtain an ample amount, the skin specimens were de-paraffinized by xylene and ethanol, then sealed in a polyethylene bag after their dry weight was measured. The specimens in the polyethylene bag were irradiated for 1 h in a pneumatic tube (Pn-2) of KUR (Kyoto University Reactor), with a thermal neutron flux of 2.8×10^{13} n per cm^2 per s to convert ^{75}As to ^{76}As. We measured γ-rays derived from ^{76}As at 559.1 keV 4–5 d after irradiation by germanium semiconductor detector (efficiency: 18%, peak to compton: 51/1, total active volume: 90 ml) (EG & G Ortec). Geometry and counting efficiency were determined before measurement by the standard elements. In practical measurements, a known amount of the standard solution was dropped onto a Millipore filter, HAWP04700 (Nihon Millipore K.K., Tokyo), irradiated, and then used as a flux monitor. The concentration of the element in the samples was calculated from the radioactivity in reference to the standard and expressed in micrograms per g of dry weight (ppm).

RESULTS

Detection of 8-OHdG in arsenic-related and arsenic-unrelated skin neoplasms Grade of 8-OHdG staining was expressed as –, +, and ++ according to the 8-OHdG index as described in *Materials and Methods*. **Figure 1(*d–f*)** shows representative staining of ++, +, and –, respectively. Thirteen of 17 cases of arsenic-related epidermal neoplasms of non-sun-exposed area and nine of 11 cases of arsenic-related epidermal neoplasms of less sun-exposed area were positively stained by monoclonal antibody N45.1 (**Fig 1**, **Table I**). On the contrary, only one of 11 samples of the arsenic-

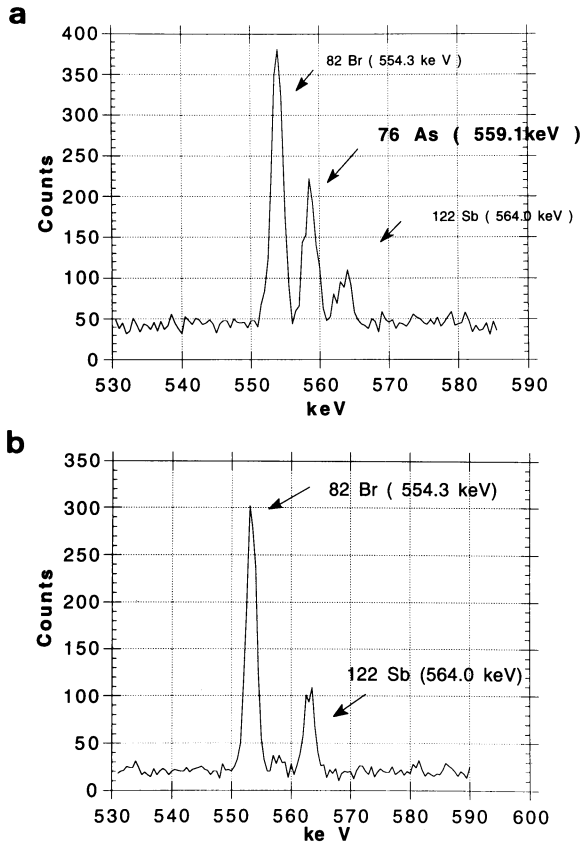

Figure 3. Detection of ^{76}As in the arsenic related skin tumors by INAA. (*a*) ^{76}As is detected from deparaffined samples of arsenic related Bowen's disease at the 559.1 keV. (*b*) ^{76}As is not detected from de-paraffinized samples of the arsenic unrelated Bowen's disease.

Table III. Arsenic detection by INAA in skin tumors

Case no.	^{76}As (ppm)
Samples from arsenic-related skin tumors[a]	
18	0
21	61.8
23	17.1
25	2.72
28	5.71
Samples from arsenic-unrelated tumors[b]	
1	0
3	0
6	0

[a]Case numbers correspond to those of **Table I**.
[b]Case numbers correspond to those of **Table II**.

unrelated Bowen's disease were stained by this monoclonal antibody ($p < 0.001$ by χ^2 test) (**Table II**). Clinicopathologic data of the tumor, and the intensity of 8-OHdG staining (8-OHdG index) are summarized in the **Tables I** and **II**.

In order to confirm the specificity of immunostaining of N45.1, monoclonal antibody against 8-OHdG, adsorption tests were performed by adding 200 and 500 μM 8-OHdG along with primary antibody on glass slides. As shown in **Fig 1(*g*)** staining color has disappeared when 8-OHdG was added during the incubation period of primary antibody.

In the positively stained samples, there was little heterogeneity in the staining pattern among the areas and almost all the cells were stained in a section. Moreover, in some samples 8-OHdG could also be detected in the nontumorous surrounding area of the epidermal cells or preneoplastic lesions (**Fig 2**). There was no correlation between the grade of malignancy (e.g., the number of

mitotic cells) and the 8-OHdG signal intensity (**Table I**). Furthermore, 8-OHdG was detected in the arsenic keratosis in the similar intensity and frequency to those of arsenic-related Bowen's disease (**Figs 1** and **2, Table I**). There was no correlation between the source of the tumor and signal intensity of 8-OHdG staining. In control samples, only 9% (one of 11) were positively stained with monoclonal antibody N45.1 (**Table II**).

Detection of arsenic in lesions by INAA In arsenic-related Bowen's disease, arsenic was detected in four of five cases from de-paraffinized sample sections(**Fig 3** and **Table III**). In contrast, in arsenic-unrelated Bowen's diseases, no arsenic was detected in three of three cases (**Table III**).

DISCUSSION

In the present study, we found significantly higher levels of 8-OHdG in the epidermal nuclei of arsenic-related Bowen's disease, Bowen's carcinoma and arsenic keratosis compared with non-arsenic-related Bowen's disease and Bowen's carcinoma (**Table I**) ($p < 0.001$). Though experimental animal models of arsenic-induced cancer have not been successfully developed (IARC, 1980), there are several findings which suggest a relationship between ROS and arsenic. Dimethylarsenic acid was shown to induce DNA damage via formation of $O_2^{-\bullet}$ and dimethylarsenic-peroxyl radical (Yamanaka *et al*, 1989, 1990). Recently a significant increase in formation of 8-OHdG in the livers of rats after administration of dimethylarsenic acid was reported (Wanibuchi *et al*, 1997). Our observation supports their results that 8-OHdG is generated by arsenic incorporation and suggests the involvement of ROS in the arsenic-induced human skin carcinogenesis as well.

There are several lines of evidence to suggest the involvement of oxidative stress in carcinogenesis. Oxidative stress can be involved in either initiation, promotion, or progression (Guyton and Kensler, 1993; Kaikobad *et al*, 1997). 8-OHdG is usually detected in target organs in high levels in experimental animal models of ROS-induced carcinogenesis (Toyokuni *et al*, 1994). It was reported that there was an increase in 8-OHdG level in the urine of cancer patients (breast, lung, colon, malignant lymphoma, malignant teratoma) as compared with healthy individuals (Tagesson *et al*, 1995). The G:C to T:A transversions, which can be induced by the presence of 8-OHdG at DNA replication, is found in the *RAS* gene and *p53* gene in human skin cancers of sun-exposed areas (Pierceall *et al*, 1991; Basset-Seguin *et al*, 1994), and UV-induced mice skin cancer (Nishigori *et al*, 1994) as well as transitions at dipyrimidine sites, signature of pyrimidine dimer presence. These imply 8-OHdG as one of the causes of solar radiation carcinogenesis. Increased levels of 8-OHdG in lymphoblasts from Fanconi's anemia, a cancer-prone disease, was also reported (Takeuchi and Morimoto, 1993). ROS induced by arsenic may work as a promoter for cells which have already been initiated by other carcinogens as suggested by Wanibuchi *et al* (1997). Persistent oxidative stress in cancer (Toyokuni *et al*, 1995) may also cause activation of transcription factors and protooncogenes such as c-*fos* and c-*jun* (Crawford *et al*, 1988) as well as genetic instability. Such a stress may also contribute toward maintaining their malignant characteristics of arsenic-related neoplasms.

The destiny of arsenic incorporated into the human body has not yet been clarified. Arsenic circulating in the blood binds to proteins by formation of a covalent complex with sulfhydryl groups of the amino acid. Cysteine in keratin is one of the major sites for accumulation of arsenic (Hindmarsh and McCurdy, 1986). To clarify more directly whether ROS formation is caused by arsenic, we examined arsenic in the lesions using INAA and revealed that at least in some specimens, arsenic remains in the skin for many years after ingestion. Miki *et al* (1982) reported that neutron activation analysis of hair showed slightly higher arsenic values in patients with arsenic-induced Bowen's disease than normal controls. Imamura and Odaka (1962) also detected arsenic in significantly higher amounts in two lesions from arsenic-related Bowen's disease than their adjacent nontumorous tissues 20 y after the apparent arsenic exposure.

Although we could not obtain the specimens for arsenic detection from nontumorous adjacent tissue in patients with arsenic-related Bowen's disease, 8-OHdG was detected not only in the tumor tissue but also in adjacent normal tissue in some cases where normal adjacent tissue was available for examination. 8-OHdG was detected in arsenic-related Bowen's disease and Bowen's carcinoma as well as in arsenic keratosis, which is generally considered to be a precancerous status. There was no correlation between the grade of malignancy and formation of 8-OHdG. Recently, the importance of the glutathione S-transferase supergene family, ROS detoxifying enzyme, is shown for the susceptibility of the individuals to various cancers including nonmelanoma skin cancer (Lear *et al*, 1997). Arsenic might inactivate these ROS detoxifying enzymes, or individuals whose ROS detoxifying enzyme activity is low, might accumulate high levels of ROS leading to arsenic-induced neoplasm. Our observations imply that arsenic remaining in the skin could contribute to the increased levels of oxidative stress for many years which could be one of the mechanisms of arsenic-induced carcinogenesis.

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